

## Effects of hypoxia acclimation on morpho-physiological traits over three generations of *Daphnia magna*

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### Summary

The mechanisms, dynamics and effects of hypoxia acclimation were studied in the water flea *Daphnia magna* over three successive generations (parental, first and second filial generation: P, F<sub>1</sub> and F<sub>2</sub>). The P generation was raised under normoxic conditions at 20°C and became exposed to environmental hypoxia (10–19% air saturation) at maturity. Their progenies (F<sub>1</sub> and F<sub>2</sub>) experienced hypoxia from birth onwards. Controls were kept under normoxic conditions. Individuals were successively sampled in a 3-day interval from each acclimation group to determine morpho-physiological parameters relevant in oxygen transport and regulation. Hypoxia acclimation induced adjustments at the haemoglobin (Hb) and metabolic level (within 3 days) but none at the systemic level. The convective performance and oxygen-sensitive control of the ventilatory and circulatory systems were the same in both acclimation

groups. The Hb concentration and oxygen affinity increased by 266% and 32%, respectively. The 22% decrease in mass-specific oxygen consumption rate reduced the energy allocation to somatic growth without greatly affecting reproduction. The onset and duration of hypoxic exposure during ontogenesis have had a significant influence on Hb oxygen affinity and body size. Transgenerational effects of hypoxia acclimation could not be observed. The adjustments at the Hb and metabolic levels in combination with the smaller body size, which is advantageous to diffusive oxygen transport, reduced the critical ambient oxygen tension by approximately 50%.

Key words: Crustacea, Branchiopoda, *Daphnia magna*, acclimation, circulation, growth, hypoxia, haemoglobin, NADH, oxygen transport, respiration, ventilation.

### Introduction

In coping with variations in ambient oxygen tension ( $P_{O_{2amb}}$ ), herbivorous zooplankters of the genus *Daphnia* are confronted with a unique set of biotic constraints arising from obligate adaptations to a competitive and predation-risky pelagic environment. First, their filter-feeding mode of life eliminates almost any possibility of a ventilatory compensation, because nutritive rather than respiratory needs have the primary control over the rate at which ambient medium passes the filtering and respiratory structures (Pirow and Buchen, 2004). Only under food-rich conditions, by relaxing the nutritive drive on limb beating activity, are animals able to respond to changes in  $P_{O_{2amb}}$  in a water breather-typical manner (cf. Dejours, 1981). Second, transparency as an anti-predation strategy is incompatible with high haemoglobin (Hb) concentrations, which cause a conspicuous body coloration (Confer et al., 1978). This constraint, however, disappears under conditions that impede visual predation or are intolerable for planktivorous fish (Hartleb and Haney, 1998). Finally, the small body size in itself poses a regulatory problem, because oxygen molecules do not exclusively follow the pathway predetermined by the circulating haemolymph. A considerable diffusive bypass exists (Pirow and Buchen, 2004; Pirow et al., 2004), which may limit

the efficiency of convective (circulatory) mechanisms in preventing body tissues from becoming ‘flooded’ with oxygen when ambient oxygen availability is high.

Given these constraints on the oxygen transport systems, the question arises as to how filter-feeding zooplankters can ensure a certain degree of oxygen homeostasis under a wide range of  $P_{O_{2amb}}$ . Oxygen homeostasis here refers to the physiological ability to *minimize* environmentally induced perturbations in internal (steady-state) oxygen levels while ensuring an adequate flow of oxygen to the tissues. It provides the twofold advantage of maintaining aerobic metabolism (as the most efficient mode of fuel utilization) and minimizing the risk of oxidative stress to tissues. There are various ways of controlling perturbations in biological systems (cf. Jones, 1973). Recent studies on the euryoxic zooplankter *Daphnia magna* have revealed two principally different regulatory mechanisms, negative feedback and buffering. Negative feedback is inherent in the circulatory and ventilatory systems as reflected by the inverse relationship between heart/limb beating rate and  $P_{O_{2amb}}$  (Paul et al., 1997; Pirow and Buchen, 2004). ‘Oxygen buffering’ (Jones, 1972) refers to the stabilizing effect occurring when haemoglobin (Hb) present in

high concentration is reversibly loaded and unloaded along the steep part of its oxygen equilibrium curve (Pirow, 2003; Pirow et al., 2004).

Chronic exposure of *D. magna* to environmental hypoxia induces gene expression-mediated adjustments in Hb concentration and oxygen affinity (Zeis et al., 2003a). This acclimatory response improves the quality of oxygen regulation under conditions of protracted ambient oxygen deficiency (Pirow et al., 2001). There are indications that hypoxia acclimation in *Daphnia* sp. causes additional changes in other characteristics such as metabolic rate (fig. 5 of Kobayashi and Hoshi, 1984; Wiggins and Frappell, 2000) and body size (Kobayashi, 1982), which may contribute to an improved hypoxia tolerance. The aim of the present study is to investigate the full spectrum and the extent of hypoxia-induced acclimatory changes by measuring all morpho-physiological parameters relevant in oxygen transport and regulation. In addition, the dynamics and possible transgenerational effects of hypoxia acclimation were studied. Finally, we tested whether the transition to and maintenance of the low-oxygen acclimation state is associated with costs reducing the reproductive success of the animal.

## Materials and methods

### *Animals and culturing conditions*

Animals of *Daphnia magna* Straus were cultured in 2 l glass beakers under normoxic conditions (86–97% air saturation,  $P_{O_{2amb}}=17.7\text{--}20.0$  kPa) and 20°C under a 16 h:8 h L:D photoperiod. The culture medium (M4; Elenndt and Bias, 1990) additionally contained  $7\ \mu\text{mol l}^{-1}$   $\text{FeSO}_4$  and had a pH of 8.2, a salinity of 0.2‰, and a conductivity of  $840\ \mu\text{S cm}^{-1}$ . Three-quarters of the medium was renewed once weekly. Animals were fed with *Desmodesmus subspicatus* at food-rich conditions ( $0.1\ \text{mg C animal}^{-1}\ \text{day}^{-1}$ ). Offspring were removed within 24 h after release from the brood chamber. Acclimation experiments were carried out under normoxic and hypoxic conditions. Hypoxic conditions (10–19% air saturation,  $P_{O_{2amb}}=2.1\text{--}3.9$  kPa) were established in 3 l preserving jars by reducing the atmospheric pressure in the residual air space to 15% of the standard pressure using a vacuum pump (Pirow et al., 2001).

### *Selection of animals and experimental schedule*

To obtain synchronized clonal animals, 26 parthenogenetic offspring of the third brood of a single female were raised under normoxic conditions. The third brood of these offspring was selected for the experiments and represented the parental (P) generation. The P generation was raised in several batches of 70–80 animals under normoxic conditions. When the first eggs appeared in the brood chamber at age 8 days, the P generation was divided and exposed to the two acclimation conditions (Fig. 1). Starting at age 8 days, batches of 8–15 individuals were successively sampled from each acclimation group in the initial phase (i.e. within the first third) of each of the first five reproductive cycles for the measurements. A sixth

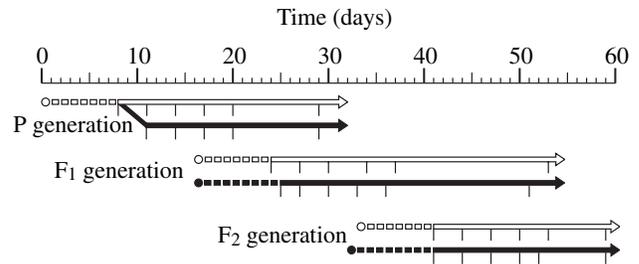


Fig. 1. Experimental schedule. Time of birth (circles) and subsequent period of life of each generation (P, F<sub>1</sub>, F<sub>2</sub>: parental, first and second filial generation, respectively; horizontal arrows). Broken lines indicate the juvenile period. White and black lines, arrows and circles indicate normoxic and hypoxic culturing conditions, respectively. Vertical ticks mark the times at which individuals were sampled from the respective generations.

measurement followed some reproductive cycles later. A single reproductive cycle was regarded to be initiated by the release of eggs into the brood chamber. The first (F<sub>1</sub>) and second filial (F<sub>2</sub>) generation were derived from the third brood of the respective parental generation.

Sampled females carried parthenogenetic embryos of developmental stage 1–2 (Green, 1956). The selection of females according to the developmental stage of their embryos ensured that the animals were always in the same stage of their moulting cycle (i.e. within 24 h after ecdysis). The embryos were removed by flushing the brood chamber with a thinly drawn-out glass capillary. The number of embryos was counted, and the carapace length ( $L_c$ , mm) of the mothers was measured as the distance between the anterior and posterior carapace margin. In addition, the distance between the base of the apical spine and the anterior part of the head was determined to derive a relationship between  $L_c$  and body length  $L_b$  ( $L_b=1.108L_c+0.157$ ,  $r^2=0.99$ ,  $N=341$ ).  $L_c$  was further used to derive the dry mass ( $W$ ,  $\mu\text{g}$ ) according to the relationship  $\ln W=3.05+2.16\ln L_c$  (Kawabata and Urabe, 1998). The debrooded mothers were then kept in nutrient-free culture medium or respiratory medium (see below) for 2 h before starting the physiological measurements.

Of a sampled batch of 8–15 individuals, 3–4 animals were used in a single respiration measurement, 3–4 animals were used to obtain one pooled sample of haemolymph for the determination of Hb concentration and oxygen affinity, and two individuals were used to analyse the ventilatory–circulatory characteristics.

### *Respiration measurements*

Oxygen consumption rate of a group of 3–4 animals was measured at  $20.0\pm 0.1^\circ\text{C}$  in a 1.5 ml closed respirometer (DW1; Hansatech instruments, Reutlingen, Germany). The respiratory medium consisted of M4-medium containing  $12.75\ \text{mmol l}^{-1}$  HEPES-buffer (pH  $8.0\pm 0.1$ ) as well as Tetracyclin and Streptomycin ( $12.5\ \text{mg l}^{-1}$  each). The chamber was cleaned every day using 70% ethanol. For calibration, the medium in the chamber was equilibrated with air and normocapnic

nitrogen. The respiratory activity of the animals and dependence on  $P_{O_{2amb}}$  was determined by monitoring the decline in oxygen concentration from normoxia to anoxia. At the end of the experiment, animals were removed without exchanging the chamber medium, and a second calibration and blank measurement followed. The oxygen consumption rate at different  $P_{O_{2amb}}$  values was obtained from small decrements in oxygen concentration per increment in time. After taking the blank measurement into account, the mass-specific oxygen consumption rate ( $\dot{M}_{O_2}$ ,  $\text{nmol h}^{-1} \text{mg}^{-1}$ ) was obtained by dividing the whole-animal oxygen consumption rate ( $\dot{m}_{O_2}$ ,  $\text{nmol h}^{-1}$ ) by dry mass of the animals.

#### Concentration and oxygen affinity of haemoglobin

A pooled haemolymph sample (1.5–2.5  $\mu\text{l}$ ) was drawn from 3–4 animals as previously described (Pirow et al., 2001) and aspirated into a glass capillary (inner and outer diameter: 0.3 mm i.d., 0.7 mm o.d.; Hilgenberg, Malsfeld, Germany). The capillary was transferred into the light path of a monolithic miniature spectrometer (MMS-UV/VIS, spectral range: 194–738 nm, 256 pixel photodiode array; Carl Zeiss, Oberkochen, Germany). The spectrometer was connected *via* Front End Electronics and a PC interface board (14 bit resolution; tec5 AG, Oberursel, Germany) to a computer (Becher, 2002). Absorption spectra (520–590 nm) were acquired (SDAS\_32D software, tec5 AG) under oxygenated conditions using water as reference. The haem-based Hb concentration was determined according to Lambert–Beer's law taking an appropriate correction for the optical path length of the cylindrical capillary into account (Becher, 2002).

Oxygen equilibria were determined on 1  $\mu\text{l}$  haemolymph samples in a diffusion chamber linked to mass flow controllers (Tylan FC280/FC260; General TCA GmbH, Eching, Germany). The chamber was positioned in the light path of the miniature spectrometer. A drop of silicon oil (AK 350; Wacker Silicone, München, Germany) covered the sample to avoid dehydration. The sample was equilibrated with gas mixtures of 12 different oxygen tensions (Fig. 4F), which were obtained by mixing air with normocapnic nitrogen. The oxygen tension was checked by an oxygen analyzer (S3A-II Ametek; Thermox instruments division, Pittsburgh, USA). Oxygen saturations were calculated from the absorption spectra as previously described (Pirow et al., 2001). The half-saturation oxygen tension ( $P_{50}$ ) and Hill's cooperativity coefficient were obtained by fitting a sigmoid curve to the oxygenation data using the Hill equation (Stryer, 1995).

#### Analysis of circulatory and ventilatory activity

Animals were tethered (Pirow et al., 2001) and transferred into a 20°C thermostatted perfusion chamber. The flow rate of the perfusion medium was set to 5  $\text{ml min}^{-1}$ . The chamber was initially perfused with air-saturated medium, and the animal was allowed to acclimate to the experimental conditions for 45 min. The oxygen tension of the perfusion medium was then gradually lowered (Freitag et al., 1998) using 19 oxygen steps with a duration of 4 min per step (Fig. 3B–E). During this

experiment, heart rate, appendage beating rate, oxygen saturation of Hb in the heart region, and the NADH fluorescence intensity of the appendage muscles were simultaneously measured as previously described (Pirow et al., 2001). The original set-up was slightly modified by using a CCD line (Proscan elektronische Systeme GmbH, Lagerfeld, Germany) for the Hb measurement and a 50 W mercury lamp (HBO 50W/AC; Osram, München, Germany) as UV light source. To avoid a continuous exposure to UV light, a triggered mechanical shutter blocked the excitation light path inbetween the measurements.

Stroke volume ( $V_s$ ) was determined from the median cross-sectional areas of the heart (in lateral view) at maximum contraction and dilation ( $A_{\text{sys}}$  and  $A_{\text{dia}}$ ,  $\text{mm}^2$ ) as described previously (Bäumer et al., 2002).  $A_{\text{sys}}$  and  $A_{\text{dia}}$  served to estimate the systolic and diastolic volumes ( $V_{\text{sys}}$  and  $V_{\text{dia}}$ , nl) using the regression equations  $\ln V_{\text{dia}} = 6.520 + 1.47 \ln A_{\text{dia}}$  and  $\ln V_{\text{sys}} = 6.541 + 1.48 \ln A_{\text{sys}}$  (modified from Bäumer et al., 2002).  $V_s$  was calculated by subtracting  $V_{\text{sys}}$  from  $V_{\text{dia}}$ .

#### Statistics

Unless otherwise stated, data are expressed as mean values  $\pm$  standard deviation (s.d.) with  $N$  indicating the number of animals examined. Differences in the body size-dependence of stroke volume were assessed by an analysis of covariance (ANCOVA). Comparisons between the two acclimation groups were performed over all generations as well as within each generation by a nonparametric two-sample rank test (Mann–Whitney test). Within each acclimation group, intergenerational differences were checked by an analysis of variance by ranks (Kruskal–Wallis test). In the case of a statistical significant difference, multiple comparisons among pairs of rank sums (Dunn's test) were used to determine between which generations the differences existed. Linear regression analysis (Zar, 1999) was used to assess the body size-dependence of a morpho-physiological variable. Statistical differences were considered as significant at  $P < 0.05$ . All statistical analyses were performed using SigmaPlot (version 8.0; SPSS Inc.), SigmaStat (version 3.01; SPSS Inc.) and Statistica (version 6.0; Statsoft Inc.).

#### Results

Acclimation of *Daphnia magna* to environmental hypoxia ( $P_{O_{2amb}} = 2.1\text{--}3.9$  kPa) was followed over 9 weeks comprising three successive generations (parental, first and second filial generation: P, F<sub>1</sub> and F<sub>2</sub>; Fig. 1). A control group remained acclimated to normoxic oxygen conditions ( $P_{O_{2amb}} = 17.7\text{--}20.0$  kPa). At several points in time, animals were sampled from both groups to quantify morpho-physiological parameters relevant in oxygen transport and regulation. In addition, the clutch size as a fitness-related parameter was measured. Most physiological variables were determined during an experimental test, which confronted the animals with a short-term (approximately 80 min) transition from normoxia to anoxia. The results are described in relation

to the following questions. Is there an acclimation effect on a given trait? Are there any intergenerational differences? How fast is the transition to the low-oxygen acclimation state? Finally, does the body size confluence variables subjected to acclimatory change?

#### Body growth and clutch size

The carapace length increased with age (7–37 days) and ranged from 1.9 to 3.4 mm (Fig. 2). The influence of hypoxia acclimation on body growth was assessed by calculating the relative difference in the carapace length ( $L_c$ ) of both acclimation groups for each of the six reproductive cycles. These relative differences were then averaged. Hypoxia acclimation resulted in a reduction of  $L_c$  by  $0.9 \pm 3.3\%$  (mean  $\pm$  s.d. of six reproductive cycles) in the P generation, by  $11.1 \pm 2.6\%$  in the F<sub>1</sub> generation, and by  $9.7 \pm 3.3\%$  in the F<sub>2</sub> generation (Fig. 2G–I). The number of embryos per brood increased with  $L_c$  without showing any clear influence of hypoxia acclimation (Fig. 2D–F). The cumulative number of eggs per animal during the first five reproductive cycles was 77 and 74 (normoxia-acclimated vs hypoxia-acclimated) in the P generation, 62 and 56 in the F<sub>1</sub> generation, and 61 and 57 in the F<sub>2</sub> generation. The reproductive success of the F<sub>1</sub>/F<sub>2</sub> generations was accordingly lower than that in the P generation.

#### Respiratory activity

The whole-animal oxygen consumption rate ( $\dot{m}_{O_2}$ ) increased with body size (Fig. 2A–C). Comparison of the mass-specific oxygen consumption rates ( $\dot{M}_{O_2}$ ) of both acclimation groups at nearly normoxic conditions ( $P_{O_{2amb}}=18$  kPa) revealed a 21% lower value in the hypoxia-acclimated animals (Table 1; Fig. 3A). Significant differences between both acclimation groups occurred in the P and F<sub>2</sub> generations (Fig. 4A).

#### Stroke volume

Diastolic and systolic heart volumes of the differently sized animals were in the range of 15–79 nl and 6–49 nl, respectively, and translated into stroke volumes ( $V_s$ ) of 4–29 nl. Hypoxia acclimation had no effect on the linear relationship between  $\ln V_s$  and  $\ln L_c$  (Fig. 5; ANCOVA: d.f.=1,  $F=0.05$ ,  $P=0.82$ ). The observed difference in the mean  $V_s$  between both acclimation groups (Table 1) is therefore explained by the depressing effect of hypoxia acclimation on  $L_c$ .

#### Concentration and oxygen-binding properties of haemoglobin

The haem-based Hb concentration increased by 266% as a consequence of hypoxia acclimation (Table 1). Significant differences between both acclimation groups occurred in all

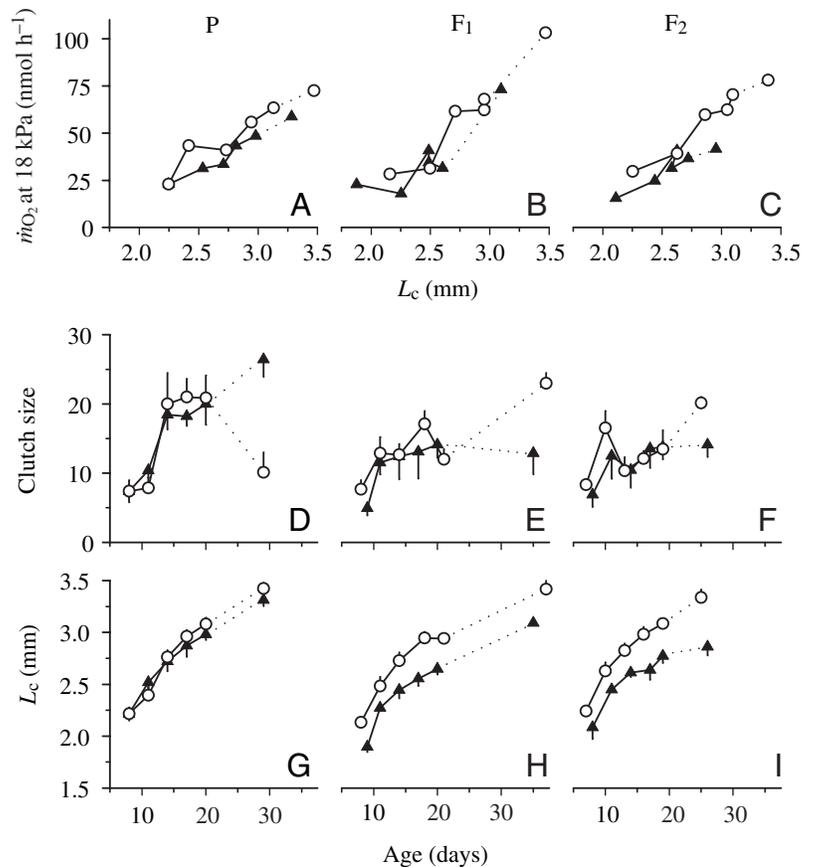


Fig. 2. Whole-animal oxygen consumption rate ( $\dot{m}_{O_2}$ ; A–C) relative to carapace length ( $L_c$ ), and age-dependence of clutch size (D–F) and carapace length ( $L_c$ ; G–I) of normoxia-acclimated (open circles) and hypoxia-acclimated animals (filled triangles). P, parental generation; F<sub>1</sub>, first filial generation; F<sub>2</sub>, second filial generation. Each data point refers to a single measurement (A–C) or represents the mean  $\pm$  s.d. of 8–15 animals (D–I).

generations (Fig. 4B). In addition, the half-saturation oxygen tension (*in vitro*  $P_{50}$ ) decreased by 32% (Table 1; Fig. 3F). Again, differences between both acclimation groups occurred in all generations (Fig. 4C). Intergenerational differences were only detectable in the hypoxia-acclimated group (Kruskal–Wallis test:  $P=0.02$ ). Multiple comparisons revealed a difference between the P and the F<sub>2</sub> generation (Dunn's Method:  $P<0.05$ ; Fig. 4C). The Hill's cooperativity ( $n_{50}$ ) coefficient decreased by 8% as a result of hypoxia acclimation (Table 1).

#### In vivo oxygenation of Hb

The ambient oxygen tension effecting a half saturation of Hb in the heart region (*in vivo*  $P_{50}$ ) decreased by 35% as a consequence of hypoxia acclimation (Table 1, Fig. 3E). Significant differences between both acclimation groups occurred in all generations (Fig. 4K).

#### Appendage beating rate

Exposing the two acclimation groups to normoxic conditions revealed a 19% lower appendage beating rate ( $f_A$ )

Table 1. Morpho-physiological characteristics of normoxia-acclimated and hypoxia-acclimated animals

Parameter	Animal acclimation group		Dependence	
	Normoxia	Hypoxia	Acclimation	$L_c$
Carapace length $L_c$ (mm)	2.85±0.37 (17)	2.63±0.35 (17)		see Fig. 2
Number of embryos	14.5±5.0 (17)	13.8±5.0 (17)		see Fig. 2
Stroke volume $V_s$ (nl)	15.3±6.0 (34)	12.3±5.0 (34)		see Fig. 5
$\dot{M}_{O_2}$ at 18 kPa (nmol h <sup>-1</sup> mg <sup>-1</sup> )	268±39 (17)	210±45 (17)	***	
Hb concentration (μmol l <sup>-1</sup> )	130±29 (17)	476±107 (17)	***	+
<i>In vitro</i> $P_{50}$ (kPa)	1.09±0.17 (16)	0.74±0.18 (16)	***	+
Hill coefficient	2.10±0.21 (17)	1.93±0.15 (17)	*	
Appendage beating rate $f_A$ (min <sup>-1</sup> )				
At normoxia	287±65 (34)	232±71 (32)	**	
At $P_{c,A}$	299±26 (34)	318±43 (34)	*	+
Heart rate $f_H$ (min <sup>-1</sup> )				
At normoxia	181±30 (34)	188±25 (34)		+
At $P_{HP}$	346±42 (34)	344±29 (34)		
At $P_{c,H}$	379±36 (34)	355±26 (34)	***	+
Indicators of oxygen tolerance				
$P_{HP}$ (kPa)	6.1±0.7 (34)	6.2±0.8 (34)		
$P_{c,O}$ (kPa)	4.3±1.0 (17)	2.0±0.6 (17)	***	
$P_{c,N}$ (kPa)	2.7±0.5 (32)	1.6±0.4 (34)	***	+
<i>In vivo</i> $P_{50}$ (kPa)	2.9±0.4 (34)	1.9±0.3 (34)	***	
$P_{c,H}$ (kPa)	1.4±0.3 (34)	1.1±0.3 (34)	***	+
$P_{c,A}$ (kPa)	1.6±0.4 (33)	0.7±0.3 (34)	***	

The data show the means ± S.D of all animals of all generations (P, F<sub>1</sub> and F<sub>2</sub>) except for carapace length  $L_c$  and number of embryos, for which the data give the means ± S.D of the means of the sampled batches. The numbers of animals and batches, respectively, are indicated in parentheses. The data of the P generation at age 8 days were excluded from the statistical comparison.

Asterisks indicate significant differences between the two acclimation groups (\* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001). A significant ( $P$ <0.05) linear relationship between a physiological trait and carapace length ( $L_c$ ) is marked by a plus sign.

in the hypoxia-acclimated group (Table 1; Fig. 3C). Significant differences between both acclimation groups occurred in the F<sub>1</sub> and F<sub>2</sub> generations (Fig. 4D). Intergenerational differences were only present in the normoxia-acclimated group (Kruskal–Wallis test:  $P$ <0.01). Multiple comparisons revealed a significant difference between the P and F<sub>2</sub> generations (Dunn's Method:  $P$ <0.05). The exposure to a gradual transition from normoxia to anoxia did not induce a counteracting response in  $f_A$  (Figs 3C, 4E), except for the hypoxia-acclimated animals, which showed a slight hyperventilation below ~2 kPa with a maximum  $f_A$  at 0.7 kPa.

#### Heart rate

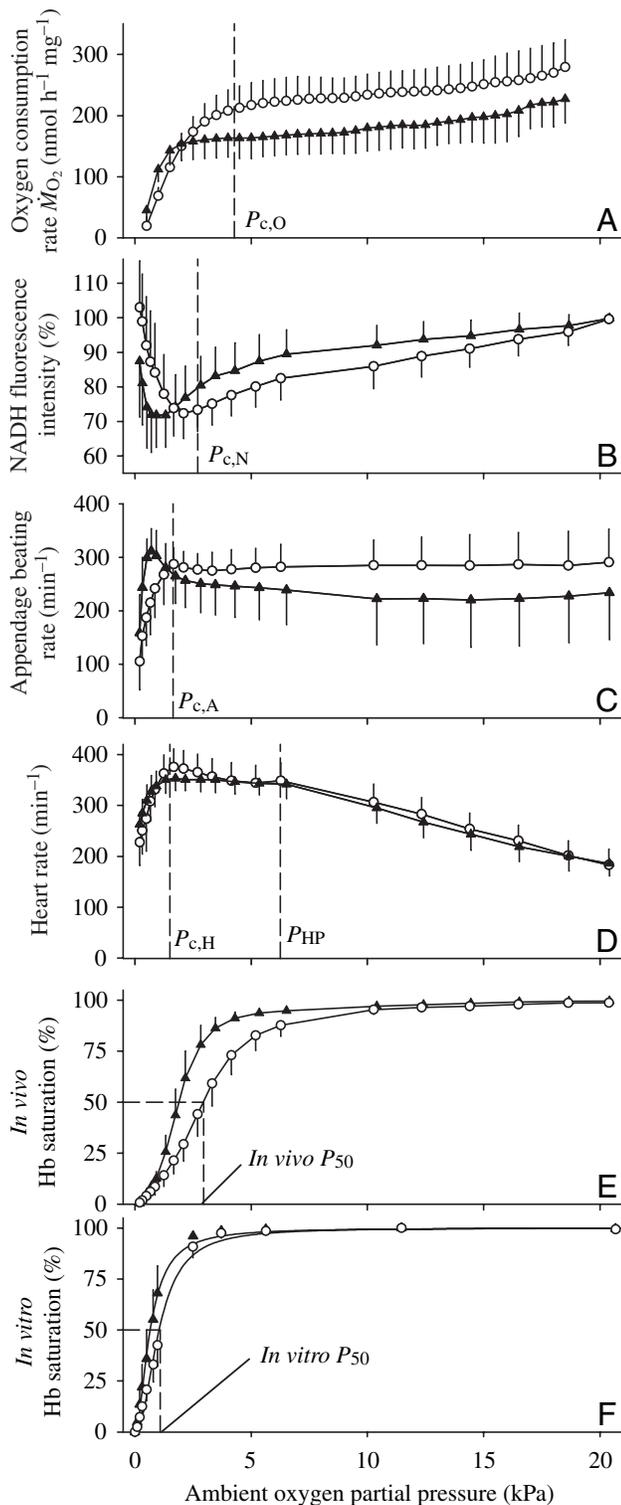
The exposure to progressive hypoxia induced a counteracting response in heart rate ( $f_H$ ), indicating the presence of an oxyregulatory (feedback) mechanism. Its operating range was the same in both acclimation groups as reflected by the identity of the lower threshold ( $P_{HP}$ =6.1–6.2 kPa; Table 1; Figs 3D, 4I), below which the  $f_H$  reached maximal values. Except at extreme values of  $P_{O_{2amb}}$  (1–2 kPa), the  $f_H$  of both acclimation groups was essentially the same, indicating the absence of an acclimation effect (Fig. 4F–H).

#### Indicators of hypoxia tolerance

The improvement of hypoxia tolerance as a consequence of hypoxia acclimation was reflected by the reduction of critical ambient oxygen partial pressures ( $P_{c,O}$ ,  $P_{c,A}$  and  $P_{c,H}$ ) at which oxygen consumption rate, appendage beating rate and heart rate, respectively, started to decrease over-proportionally (Fig. 3A,C,D). The  $P_{c,O}$  decreased by 52% (Table 1). Significant differences between both acclimation groups occurred in the F<sub>1</sub> and F<sub>2</sub> generations (Fig. 4L). The  $P_{c,A}$  decreased by 66% (Table 1). Significant differences occurred in all generations (Fig. 4N). Intergenerational differences were only present in the normoxia-acclimated group (Kruskal–Wallis test:  $P$ =0.01). The  $P_{c,H}$  decreased by 21% (Table 1). Significant differences occurred in the F<sub>1</sub> and F<sub>2</sub> generation (Fig. 4J). The improvement of hypoxia tolerance was also signalized by the 61% reduction of  $P_{c,N}$  (Fig. 3B, Table 1), the ambient oxygen partial pressures at which the NADH fluorescence intensity of the appendage muscles started to increase. This increase indicated the impairment of tissue oxygen supply (Pirow et al., 2001). Significant differences occurred in all generations (Fig. 4M).

## Dynamic of hypoxia acclimation

The main acclimatory adjustments occurred in the P generation within the first 3 days after the start of hypoxic incubation. The fast transition to the low-oxygen acclimation state is apparent in the Hb concentration and oxygen consumption rate ( $\dot{M}_{O_2}$ ) as well as in the indicators of hypoxia tolerance such as  $P_{c,A}$  and  $P_{c,N}$  (Fig. 6).



## Body-size dependence

The linear dependence of a physiological variable on carapace length was tested in both acclimation groups over all generations (Table 1). As an exception, the P generation of the hypoxia-acclimated group was excluded because of the transient acclimatory adjustments. A significant positive dependence was found for the Hb concentration (hypoxia-acclimated:  $P=0.04$ ; Fig. 7A) and the  $P_{c,H}$  (both acclimation groups:  $P<0.01$ ; Fig. 7G). A negative dependence occurred in the *in vitro*  $P_{50}$  (hypoxia-acclimated:  $P<0.01$ ; Fig. 7B), the appendage beating rate at the  $P_{c,A}$  (both acclimation groups:  $P<0.01$ ; Fig. 7C), the  $P_{c,N}$  (normoxia-acclimated:  $P=0.03$ ; Fig. 7D), and the heart rate at normoxia and at the  $P_{c,H}$  (hypoxia-acclimated:  $P<0.01$ ; Fig. 7E,F).

## Discussion

The transfer of *D. magna* from a normoxic to a hypoxic environment induced significant acclimatory changes in only a few morpho-physiological traits relevant for oxygen regulation and tolerance. The concentration and oxygen affinity of Hb increased by 266% and 32%, whereas mass-specific oxygen consumption rate and body size decreased by 22% and 8% (Table 1). Other traits such as the correlation between body size and stroke volume (Fig. 5) remained essentially unaffected.

These acclimatory adjustments improved the hypoxia tolerance by reducing the critical ambient oxygen partial pressures ( $P_{c,O}$ ,  $P_{c,A}$ ,  $P_{c,H}$ ) at which oxygen consumption rate, appendage beating rate, and heart rate started to decrease over-proportionally (Fig. 3A,C,D). The reduction of  $P_{c,H}$  was only 21% (Table 1). The  $P_{c,O}$  and  $P_{c,A}$  decreased by 52% and 66%, a change quite similar to the 61% reduction in  $P_{c,N}$  (Table 1), which signaled the incipient impairment of tissue oxygen supply (Pirow et al., 2001).

Of the two regulatory mechanisms, negative feedback and oxygen buffering (*via* Hb), only the latter became reinforced during hypoxia acclimation whereas the former remained unaffected. Confronting differently acclimated animals with a short-term (~80 min) transition from normoxia to anoxia elicited a counteracting increase in heart rate (compensatory tachycardia; Paul et al., 1997), which indicates the presence of an active negative-feedback mechanism. Its operating range

Fig. 3. Summary of the physiological characteristics of normoxia-acclimated (open circles) and hypoxia-acclimated animals (filled triangles). The data of the three generations (P, F<sub>1</sub> and F<sub>2</sub>) were pooled and given as means  $\pm$  s.d. Also shown are the physiological responses (A–E) to decreasing ambient oxygen partial pressures ( $P_{O_{2amb}}$ ) or oxygen equilibrium curves (F) of haemolymph samples. Sigmoid curves in E and F were fitted to the data using the Hill equation. Broken lines represent critical  $P_{O_{2amb}}$  values, which indicate a deflection point on the response curve of a physiological parameter ( $P_{c,O}$ ,  $P_{c,N}$ ,  $P_{c,A}$ ,  $P_{c,H}$ ,  $P_{HP}$ ) or the half-saturation oxygen tension of Hb ( $P_{50}$ ). For clarity, the critical values are given only for normoxia-acclimated animals.

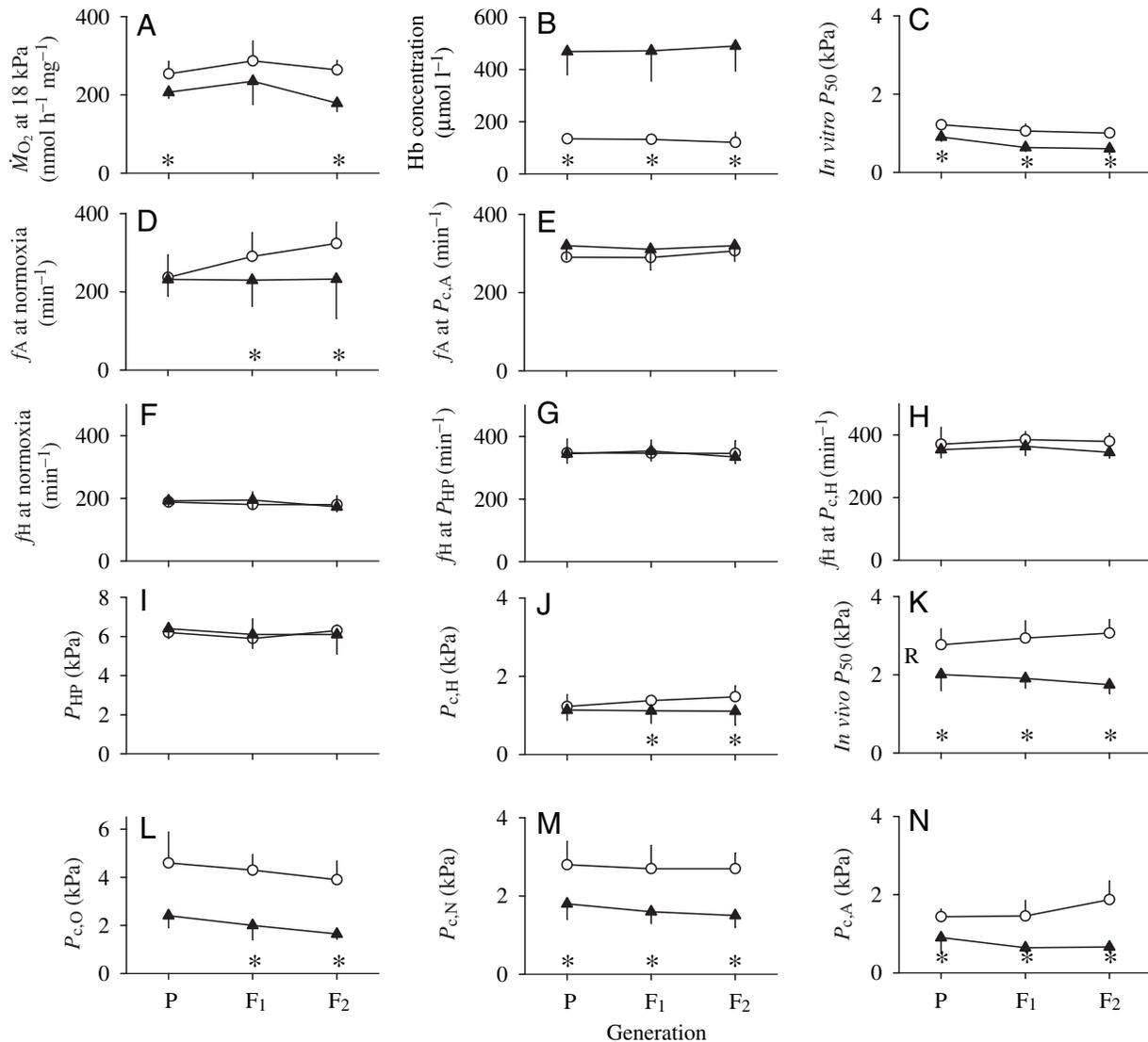


Fig. 4. Summary of the morphological and physiological characteristics of normoxia-acclimated (open circles) and hypoxia-acclimated animals (filled triangles) of the three generations (P, F<sub>1</sub> and F<sub>2</sub>). Values are means  $\pm$  S.D. The numbers of measurements on different animals per data point were 5–6 (A–C,L) and 10–12 (D–K,M,N), respectively. Asterisks indicate significant differences between the two acclimation groups within one generation (\**P*<0.05).

and the magnitude of  $f_H$  in dependence on  $P_{O_{2amb}}$  were the same in both acclimation groups (Fig. 3D), suggesting a uniform oxygen-dependent drive of heart rate (Paul et al., 2004). The appendage movement, which may assume an oxyregulatory function under food-rich conditions (Pirow and Buchen, 2004), was essentially unaffected by decreasing  $P_{O_{2amb}}$  except at extreme values of  $\sim 2$  kPa, at which hypoxia-acclimated animals developed a slight hyperventilation (Fig. 3C). These animals, when tested under normoxic conditions, also had a 19% lower appendage beating rate compared to the normoxia-acclimated group. It seems, however, unlikely that a reduction of that magnitude has a significant effect on internal oxygen levels as control analysis has shown (Pirow and Buchen, 2004).

The oxygen-buffering mechanism was reinforced during

hypoxia acclimation by adjustments at the Hb level (see below). This mechanism comes into play when the internal oxygen levels decrease to such low values that Hb is reversibly loaded and unloaded along the steep part of its oxygen equilibrium curve. As signaled by the changes in Hb oxygen saturation in the heart region (Fig. 3E), this condition (inside the animal) became satisfied when the  $P_{O_{2amb}}$  approached and fell below the lower threshold of the circulatory feedback mechanism ( $P_{HP}$ =6.1–6.2 kPa, both acclimation groups). Hypoxia acclimation broadened the operating range of the oxygen-buffering mechanism by reducing the lower threshold ( $P_{c,O}$ ) from 4.3 to 2.0 kPa. This gain of operating range is almost congruent with the range of low-oxygen conditions ( $P_{O_{2amb}}$ =3.9–2.1 kPa) to which the animals became acclimated. The adjacency of the operating ranges for oxygen buffering *via*

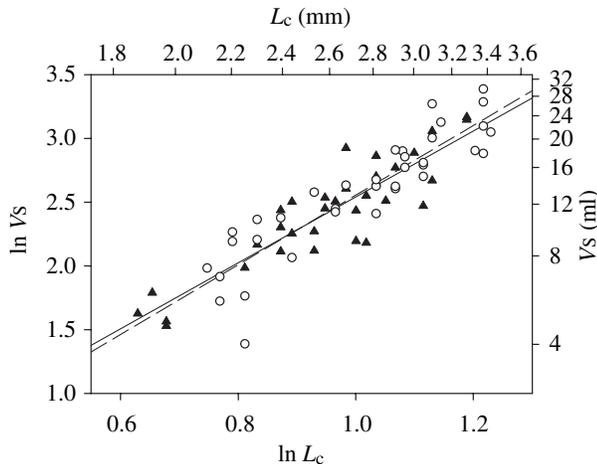


Fig. 5. Relation between logarithmically transformed stroke volume ( $\ln V_s$ ,  $V_s$  in nl) and carapace length ( $\ln L_c$ ,  $L_c$  in mm) of normoxia-acclimated (open circles) and hypoxia-acclimated animals (filled triangles) of all generations (P, F<sub>1</sub> and F<sub>2</sub>). The broken and solid lines represent linear regression lines for the data of the former ( $\ln V_s = -0.178 + 2.734 \ln L_c$ ,  $r^2 = 0.81$ ,  $N = 36$ ) and the latter groups ( $\ln V_s = -0.0454 + 2.588 \ln L_c$ ,  $r^2 = 0.80$ ,  $N = 33$ ), respectively.

Hb (2–6 kPa) and circulatory control (6–20 kPa) is an interesting example for the transition of regulatory control from one mechanism to another, which allows the animal to extend the range of tolerable ambient oxygen tensions.

#### Comparison with previous data

The haem-based Hb concentrations of normoxia-acclimated and hypoxia-acclimated *D. magna* (130 vs 476  $\mu\text{mol l}^{-1}$ ) are comparable to previous data (49–114  $\mu\text{mol l}^{-1}$  vs 337–600  $\mu\text{mol l}^{-1}$ ; Pirow et al., 2001; Zeis et al., 2003a). The *in vitro*  $P_{50}$  (1.01–1.20 kPa) measured in whole-blood samples of the normoxia-acclimated generations was similar to the value of 1.0 kPa reported by Zeis et al. (2003a). In the hypoxia-acclimated animals, this variable decreased to 0.92, 0.66 and 0.61 kPa in the P, F<sub>1</sub> and F<sub>2</sub> generations. The latter two values agree with the value of 0.6 kPa from long-term acclimated animals (Zeis et al., 2003a).

Subsequent comparisons are restricted to normoxia-acclimated animals, as most data from literature are available for animals raised under normoxic conditions. The mass-specific oxygen consumption rate ( $\dot{M}_{O_2}$ ) of debrooded, fasting (2 h) animals being in the first third of their reproductive cycle was 268  $\text{nmol mg}^{-1} \text{h}^{-1}$  under almost normoxic conditions ( $P_{O_{2amb}} = 18 \text{ kPa}$ ) and 20°C. Glazier (1991) reported a similar value of 234  $\text{nmol mg}^{-1} \text{h}^{-1}$  for debrooded, fasting (24 h) animals. The dependence of stroke volume on carapace length followed a power function similar to that given by Bäumer et al. (2002).

Upon response to a progressive reduction in  $P_{O_{2amb}}$ , our animals showed the typical responses in *in vivo* oxygen saturation of Hb, heart rate and appendage beating rate (Paul et al., 1997; Pirow et al., 2001; Pirow and Buchen, 2004). Against our expectation, the critical ambient oxygen partial

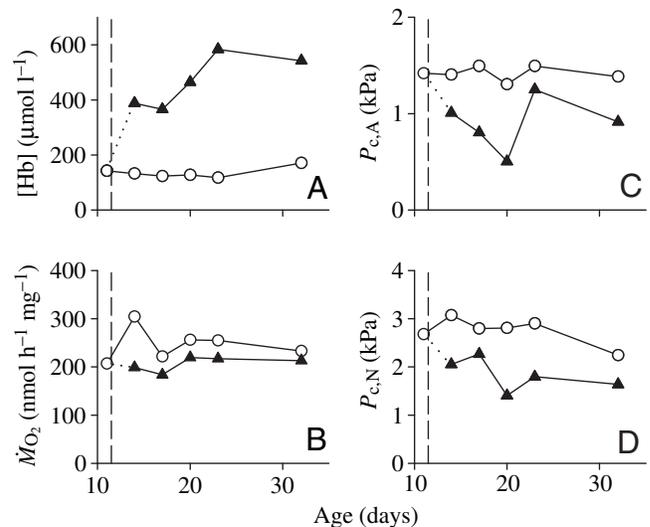


Fig. 6. Time course of selected physiological parameters of normoxia-acclimated (open circles) and hypoxia-acclimated animals (filled triangles) of the P generation. The start of hypoxic incubation is indicated by vertical broken lines. Each data point represents one measurement (B) or shows the mean of two measurements (A, C, D) on different animals. [Hb], haem-based haemoglobin concentration;  $P_{c,N}$ ,  $P_{c,A}$ , critical  $P_{O_{2amb}}$  values (see Fig. 3B, C);  $\dot{M}_{O_2}$ , mass-specific oxygen consumption rate.

pressure of tissue oxygen supply ( $P_{c,N} = 2.7 \text{ kPa}$ ) deviated from that of oxygen uptake rate ( $P_{c,O} = 4.3 \text{ kPa}$ ). The latter values agrees quite well with the  $P_{c,N}$  of 4.6 kPa reported by Pirow et al. (2001). The deviation therefore cannot be explained by the two different experimental conditions, under which the animals were either able to swim freely in a closed respirometer or tethered in a perfusion chamber. The lower  $P_{c,N}$  obtained in the present study was possibly the consequence of the replacement of the light source used for NADH excitation. Greater instabilities in light intensity might have impeded the early detection of the incipient increase in NADH fluorescence intensity under progressive hypoxia.

#### Adjustments at the Hb level

The comparison of differently acclimated *D. magna* revealed the known negative correlation between Hb concentration and half-saturation oxygen tension (Table 1; Kobayashi et al., 1988; Zeis et al., 2003a). The advantage of this inverse relationship is to minimize the total Hb concentration required to ensure the adequate supply of oxygen to the tissues under different ambient oxygen conditions (Kobayashi et al., 1994). Hb-poor animals living under moderate-to-high oxygen conditions benefit from a low-affinity Hb, which operates at a higher and more extended range of (internal) oxygen levels. Using instead a high-affinity Hb for the same range of oxygen tension would require a higher concentration to obtain the same transport/buffering capacity for oxygen. In contrast, Hb-rich animals living under low ambient oxygen conditions benefit from a high-affinity

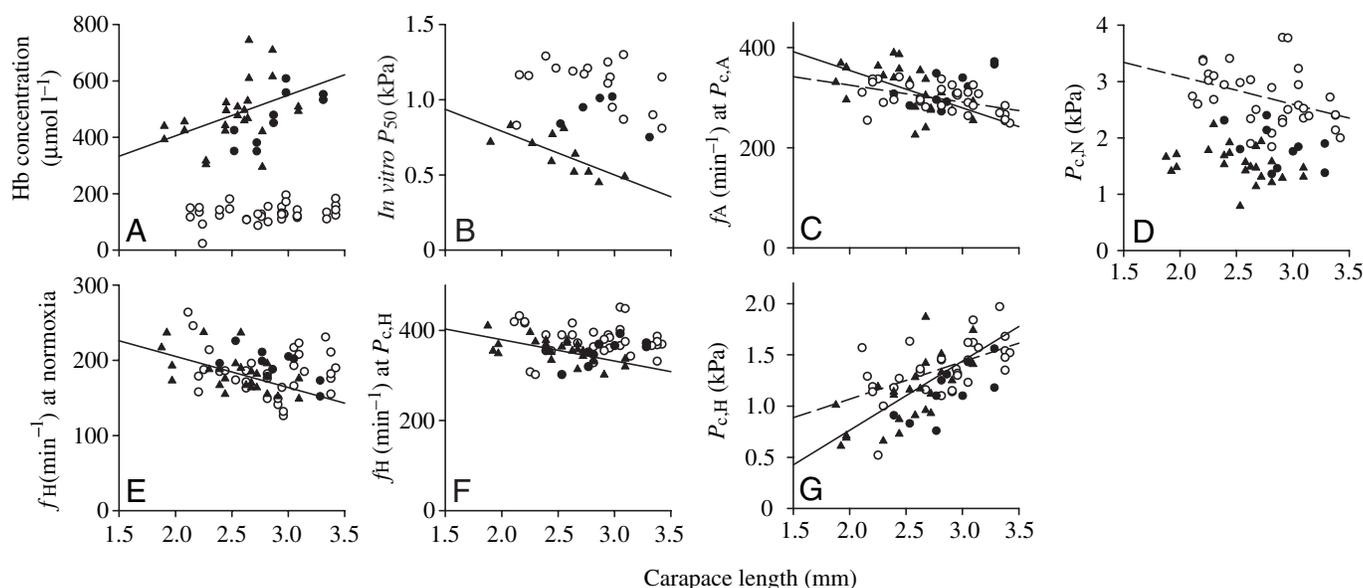


Fig. 7. Summary of all physiological parameters which showed a dependency on carapace length. Open circles represent the data of all normoxia-acclimated animals with dashed lines indicating significant linear dependencies. The data of hypoxia-acclimated animals are given separately for the P generation (filled circles) and the F<sub>1</sub> and F<sub>2</sub> generation (filled triangles). The data of both filial generations were used for linear regression analysis with solid lines indicating significant linear dependencies. Other details as in Fig. 4.

Hb. Theoretical considerations based on the analysis of finely-resolved oxygen equilibrium curves suggest that animals having a low-affinity Hb instead would need more than three times as much Hb to transport the same amount of oxygen (Kobayashi et al., 1994). As previously discussed (Bäumer et al., 2002; Paul et al., 2004), the increased oxygen affinity of Hb in hypoxia-acclimated animals might compromise the unloading of oxygen to the tissues. The reduction of oxygen consumption rate and body size mitigates this problem by lowering critical partial pressure needed to drive the diffusion of oxygen into the tissues.

#### Dynamic of hypoxia acclimation

The transfer from the normoxic to the hypoxic environment occurred when females had entered into the first reproductive cycle. Analyzing these animals 3 days later showed a considerably higher concentration of Hb compared to animals kept in parallel under normoxic conditions (Fig. 6A). The induction of Hb in *D. magna* is a fast process. Transcripts of hypoxia-inducible Hb genes occur within a few hours after starting the hypoxic incubation (Zeis et al., 2003a). Initial differences in Hb concentration are already detectable after 1 day of hypoxic incubation (Kobayashi et al., 1990), and 3 days are required to attain a stable high level (Kobayashi et al., 1990; Zeis et al., 2003b). The present study shows that the rapid induction of Hb is paralleled by a rapid reduction (within 3 days) in oxygen consumption rate (Fig. 6B). The simultaneous reduction of tolerance indicators ( $P_{c,A}$  and  $P_{c,N}$ ; Fig. 6C,D) confirm that a 3-day period is sufficient for adults to transit to a new acclimation state.

#### Metabolic rate, growth and reproduction

When tested at almost normoxic conditions ( $P_{O_2,amb}=18$  kPa), hypoxia-acclimated animals had a 22% lower (mass-specific) oxygen consumption rate ( $\dot{M}_{O_2}$ ) compared to normoxia-acclimated animals. Based on internal oxygen measurements (Pirow et al., 2004), it is reasonable to assume that under these test conditions the internal oxygen levels were relatively high and comparable in both groups. Accordingly, cellular oxygen levels cannot be responsible for the low  $\dot{M}_{O_2}$  of the hypoxia-acclimated group. Changes in metabolic rate might arise from alterations in mitochondrial density and/or capacity, which are well known to occur in animals during thermal acclimation (Pörtner, 2002). In *D. magna*, low internal oxygen levels arising from the chronic exposure to a hypoxic environment could effect intermediate-term adjustments in mitochondrial function.

The reduction of  $\dot{M}_{O_2}$  was correlated with a decrease in somatic growth rate. Hypoxia-acclimated animals had a smaller body size than similar-aged normoxia-acclimated animals (Fig. 2G–I). Both acclimation effects had already become apparent in the P generation a few days after starting the hypoxic exposure (Figs 2G,A, 6B). The growth retardation was more pronounced in the F<sub>1</sub> and F<sub>2</sub> generations, which experienced the low-oxygen conditions from birth onwards. Smaller body sizes are advantageous for diffusive oxygen-transport processes and therefore contribute to an improved hypoxia tolerance (Pirow and Buchen, 2004; Pirow et al., 2004). In contrast to somatic growth, the reproductive success remained essentially unchanged. Hypoxia-acclimated animals, although being smaller than normoxia-acclimated animals, had similar clutch sizes during the first five broods (Fig. 2D–F).

A lower  $\dot{M}_{O_2}$  implies that less energy is available for maintenance, growth and reproduction. A significant anaerobic supplementation of energy provision is unlikely because in Hb-rich animals the increase in the concentration of anaerobiosis indicators (lactate) occurs at a  $P_{O_{2amb}}$  lower than 2 kPa (Usuki and Yamagushi, 1979). The present study shows that hypoxia-acclimated animals of *D. magna* maintain reproductive performance while reducing the energy allocation to growth-related processes. The experimental data provide no obvious indications of a reduction of maintenance costs. The appendage beating rates and the duration of the moulting cycles of both groups (Figs 3C, 1) at the respective acclimation conditions (2.1–3.9 vs 17.7–20.0 kPa) were essentially the same, suggesting similar energetic expenditures for both processes.

#### Intergenerational differences

The parental environment and state can significantly impact offspring performance. In *Daphnia* sp., such maternal or transgenerational effects are described for inducible defences in response to predator-borne chemicals (Agrawal et al., 1999) and for resting egg production in response to photoperiod and food conditions (LaMontagne and McCauley, 2001; Alekseev and Lampert, 2001). Besides the possibility of transmitting information about the environment to the progenies, the maternal state itself can influence the energy allocation to the offspring, thereby altering their growth rates, survivorship and fecundity (LaMontagne and McCauley, 2001).

Following the response of *D. magna* to a new oxygen environment over three successive generations revealed intergenerational differences in Hb oxygen affinity (Fig. 4C), growth (Fig. 2G–I) and reproduction (Fig. 2D–F). The clutch size in the  $F_1/F_2$  generations was reduced in comparison to the P generation. However, this intergenerational difference occurred not only in the hypoxia-acclimated group but also in the normoxia-acclimated group. Differences in reproductive performance could arise from the fact that the mothers of the P generation were cultured in batches of 25 animals, whereas the mothers of the  $F_1$  and  $F_2$  generations were raised in batches of 70–80 individuals (for crowding effects, see Guisande, 1993; Goser and Ratte, 1994).

Body size and Hb oxygen affinity were affected by hypoxia acclimation, and both variables again assumed lower values in the  $F_1/F_2$  generation (Figs 2G–I, 4C). These intergenerational differences, however, resulted mainly from differences in the onset and duration of hypoxic exposure during ontogenesis. The  $F_1/F_2$  generations experienced the low oxygen condition from birth onwards, whereas the P generation became exposed to hypoxia after reaching maturity. The growth retardation was correlated with a reduction of  $\dot{M}_{O_2}$ , which occurred in all hypoxia-acclimated generations. The higher *in vitro*  $P_{50}$  of the P generation can be explained by the mixing of newly synthesized Hb species of high affinity with low-affinity species (Zeis et al., 2003a) that were already present in juvenile stages. Individuals of the  $F_1/F_2$  generations, in contrast, produced high-affinity haemoglobins from birth. In conclusion, a hypoxia-induced transgenerational effect

responsible for the observed intergenerational differences cannot be substantiated.

#### Body-size dependencies

In addition to the whole body oxygen consumption rate ( $\dot{m}_{O_2}$ ) and clutch size, several other variables were correlated with body size, either in one or both acclimation groups. The Hb concentration and oxygen affinity of hypoxia-acclimated animals increased with body size (Fig. 7A,B), thereby counteracting growth-related problems in internal oxygen transport. Higher concentrations of Hb are associated with an increase in haemolymph viscosity and oxygen-transport capacity, which might explain the negative correlation between heart rate and body size in hypoxia-acclimated animals (Fig. 7E,F). In both acclimation groups, appendage beating rate at the  $P_{c,A}$  decreased with body size, indicating that larger animals were unable to move the limbs at the same maximum frequency as smaller ones. Both acclimation groups also showed a positive correlation of the  $P_{c,H}$  with body size (Fig. 7G). The supply of oxygen to the heart at critical  $P_{O_{2amb}}$  therefore seems more dependent on body size than on Hb concentration.

#### Synopsis

Heart rate control and oxygen buffering *via* Hb are the main oxyregulatory mechanisms that enable *Daphnia magna* to respond immediately to fluctuating ambient oxygen levels. Conditions of protracted ambient oxygen deficiency induce intermediate-term adjustments at the Hb and metabolic levels but none at the systemic level. The main acclimatory adjustments are completed within 3 days after starting the hypoxic exposure. The expression of hypoxia-inducible Hb genes results in an increase in the concentration and oxygen affinity of Hb. The decrease in mass-specific oxygen consumption rate, which might result from mitochondrial adjustments, reduces the energy allocation to somatic growth without greatly affecting reproduction. Smaller body sizes are advantageous to diffusive processes and therefore contribute to the improved hypoxia tolerance. The onset and duration of hypoxic exposure during ontogenesis have a significant influence on Hb oxygen affinity and body size. Transgenerational effects of hypoxia acclimation could not be observed.

#### List of symbols

$A_{sys}$	median cross-sectional area of the heart at maximum contraction ( $mm^2$ )
$A_{dia}$	median cross-sectional area of the heart at maximum dilation ( $mm^2$ )
$L_b$	body length (mm)
$f_A$	appendage beating rate ( $min^{-1}$ )
$f_H$	heart rate ( $min^{-1}$ )
$L_c$	carapace length (mm)
$\dot{M}_{O_2}$	mass-specific oxygen consumption rate ( $nmol\ h^{-1}\ mg^{-1}$ )

$\dot{m}_{O_2}$	whole-animal oxygen consumption rate (nmol h <sup>-1</sup> )
$P_{50}$	half-saturation oxygen tension of haemoglobin (kPa)
$P_{c,A}$	critical oxygen partial pressure of the appendage beating rate (kPa)
$P_{c,H}$	critical oxygen partial pressure of the heart rate (kPa)
$P_{c,N}$	critical oxygen partial pressure of the NADH fluorescence intensity (kPa)
$P_{c,O}$	critical oxygen partial pressure of the oxygen consumption rate (kPa)
$P_{HP}$	ambient oxygen partial pressure at the beginning of the heart rate plateau (kPa)
$P_{O_{2amb}}$	ambient oxygen partial pressure (kPa)
$V_{dia}$	diastolic volume (nl)
$V_S$	stroke volume (nl)
$V_{sys}$	systolic volume (nl)
$W$	dry body mass (µg)

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